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13. ABSTRACT *(Maximum 200 Words)*

The experiments performed during the second grant period were designed to investigate the role of arachidonic acid (AA) release and metabolism in the toxicity caused by the depletion of GSH in primary mesencephalic cultures. Our experiments demonstrated that: (1) Inhibition of phospholipase A_2, which decreases AA release, and inhibition of lipoxygenease, which prevents AA metabolism, protect cells from the toxicity of GSH depletion. (2) We confirmed the AA release is increased during GSH depletion. (3) We showed that exogenous AA is toxic to GSH depleted cells. (5) We demonstrated that products of AA metabolism cause damage to cells, when depleted of GSH. The toxicity of AA is also prevented by inhibition of lipoxygenase, further supporting a role of AA in GSH depletion induced cell death. In summary, our studies provide evidence that depletion of GSH can increase the release and metabolism of AA and that toxic oxygen free radicals produced during AA metabolism, and in particular superoxide, are responsible for GSH depletion induced cell death. A characteristic of Parkinson's disease is GSH depletion in the brain. Our study provides information on the events that lead to toxicity and could help in the design of therapeutic intervention in Parkinson's disease.

14. SUBJECT TERMS
Parkinson; Dopamine neuron; glutathione; Oxidative stress; Cytokines

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Table of Contents

INTRODUCTION

Parkinson's disease is a progressive neurodegenerative disorder afflicting primarily the older population. The major neuropathological feature of Parkinson's disease is the degeneration of dopamine neurons in the substantia nigra pars compacta, which results in depletion of the neurotransmitter dopamine and loss of motor control. Our research goal is to understand the cause for the degeneration of dopamine neurons in Parkinson's disease. Oxidative stress is thought to play a role in the pathogenesis of Parkinson's disease and it has been proposed that the loss of the antioxidant glutathione (GSH) may be an early event in the development of the disorder. We have been studying the effects of GSH depletion in an *in vitro* model of primary cultures of embryonic rat substantia nigra. Our previous studies have demonstrated that GSH depletion causes neuronal cell death, which is mediated by glial cells. Understanding the mechanisms that regulate the glial-mediated cell death during depletion of GSH is the focus of our current studies.

BODY

The research proposed in the statement of work for the second year of the grant period included the following: (1) Completion of the ***"study of mechanisms of glial mediated cell death"***, which was initiated in the first year. (2) ***Study of the role of arachidonic acid and metabolites of the cyclooxygenase (COX) and lipoxygenase (LOX) pathways in cell death caused by GSH depletion".***

The major findings from the study of mechanisms of glial mediated cell death have been

included in a manuscript entitled "Lipopolysaccharide Up-regulates Manganese Superoxide Dismutase in Mesencephalic Cultures and Protects from Cell Death Caused by Glutathione Depletion" by Brian C. Kramer, Jocelyn A. Yabut, Thalia Robakis and Catherine Mytilineou, which has been submitted for publication and is currently under review. A copy of the submitted manuscript is appended to this report.

1. Mechanisms of glial mediated cell death

The majority of the experiments for this study were conducted during the first grant year and were reported in the previous progress report. In summary, we used the endotoxin lipopolysaccharide (LPS) to activate the glial cells in mesencephalic cultures and simulate the environment of an inflammatory response. Contrary to our expectations, LPS provided protection from cell death caused by GSH depletion (Appendix: Kramer et al., Fig. 2). A systematic study of the various ways that glial activation by LPS could protect from toxicity were examined and it was concluded that protection was not due to secretion of cytokines or growth factors (Appendix: Kramer et al., Table 2 & Fig. 4). We also demonstrated that LPS was actually causing some oxidative stress (Appendix: Kramer et al., Fig. 3) and that the cytokine interleukin 1β (IL-1β), which was increased in the LPS treated cultures, has the potential to kill cells when GSH levels are reduced (Appendix: Kramer et al., Fig. 5).

In the current grant period we examined the possibility that the mild oxidative stress caused by LPS could up-regulate antioxidant enzymes in mesencephalic cultures, which could protect from further oxidative stress. Our experiments showed that treatment with LPS increased the activity of

the mitochondrial antioxidant enzyme superoxide dismutase (Mn-SOD) (Appendix: Kramer et al., Fig. 6). The cytosolic form of the enzyme Cu,Zn-SOD was not affected by LPS treatment. We also demonstrated that the increase in Mn-SOD activity is accompanied by a significant increase in the protein content, suggesting increased enzyme synthesis (Appendix: Kramer et al., Fig. 6). Imuunocytochemistry with antibodies to Mn-SOD showed the the increase in the enzyme protein was mainly within astrocytes (Appendix: Kramer et al., Fig. 9).The ability of SOD to protect from GSH depletion-induced damage was confirmed when addition of SOD, but not catalase, to the culture medium protected from cell death caused by depletion of GSH (Appendix: Kramer et al., Fig. 7).

The significance of this study is two-fold: First it shows that ***an inflammatory response causes oxidative stress*** and that ***IL-1β which is increased in response to inflammation can cause the damage in GSH-depleted cells***. Second, we show that ***during depletion of cellular GSH the most damaging oxygen free radical is superoxide*** and that ***up-regulation of SOD can protect neuronal cells from GSH-depletion induced damage***.

The studies concerning the involvement of NMDA receptors and nitric oxide gave essentially negative results. Blockade of NMDA receptors did not protect from the toxicity cause by treatment with the inhibitor of GSH synthesis L-buthionine sulfoximine (BSO). Similarly, only partial protection could be afforded by inhibiting nitric oxide synthase (NOS) activity and this finding was always consistent, suggesting that activation of NMDA receptors and NOS activity are not major components of the events that lead to cell death.

2. Study of the role of arachidonic acid and metabolites of COX and LOX pathways in cell death caused by GSH depletion

Arachidonic acid (AA), the most common fatty acid in brain phopholipids, has been implicated in brain injury (Katsuki and Okuda, 1995). Primary cultures of astrocytes can synthesize arachidonic acid from essential fatty acid precursors, while primary neuronal cultures lack the ability to perform the desaturation steps to produce AA (Moore *et al.*, 1991). Brain neurons take up free AA by the blood through the vascular endothelial cells and by astrocytes (Katsuki and Okuda, 1995). If AA metabolism is the primary cause of damage to GSH depleted neurons, it would be expected that the presence of glial cells in the cultures should increase the toxicity of GSH depletion.

Liberation of AA from membrane phospholipids in the brain occurs primarily through tha action of phospholipase A_2 (PLA_2). Free AA can then be metabolized by the lipoxygenase, cyclooxygenase and cytochrome P450 pathways. Our previous studies indicated that metabolic products of AA by the LOX pathway are likely responsible for the toxicity caused by GSH depletion (Mytilineou *et al.*, 1999). In the studies described here we examined in more detail the role of PLA_2 and LOX products of AA metabolism on the toxicity caused by the inhibition of GSH synthesis following treatment with L-buthionine sulfoximine (BSO).

2a. Effect of PLA_2 and LOX inhibition during the course of GSH depletion on cell survival.

To determine the role of PLA_2 and LOX we treated cultures with BSO and the inhibitors of the enzymes were added at the time of exposure to BSO, 24 h later, or 30 hrs later. We have previously shown that BSO toxicity is a delayed event that usually occurs 48 h after treatment (Mytilineou *et al.*, 1999). Our experiments demonstrated that PLA_2 inhibitors (Appendix Fig. 1) as well as LOX inhibitors (Appendix Fig. 2) were able to protect cells from injury, even when applied at a very late stage (30 h after treatment). These data demonstrate that the liberation and metabolism of AA are late events, which occur shortly before the initiation of damage.

2b. Toxicity of arachidonic acid in pure neuronal and mixed cultures

Addition of AA to the culture medium at concentrations from 1-50 μM is well tolerated by mesencephalic cultures, causing no apparent damage. However, when the levels of GSH are reduced by BSO treatment, AA becomes very toxic to both mixed mesencephalic cultures (Appendix Fig. 3) and pure neuronal cultures (Appendix Fig. 4). In mixed neuronal-glial cultures the toxicity of AA can be completely prevented by inhibiting LOX with 10 μM NDGA or 5 μM biacalein (Appendix Fig. 3). In pure neuronal cultures 10 μM NDGA was very toxic by itself. However, a lower concentration of NDGA (1 μM) provided partial protection, suggesting that the toxicity of AA in the BSO treated cells was due to the metabolic products from the LOX pathway (Appendix Fig. 4). We are currently testing biacalein to clarify this point.

From these experiments we conclude that the release and metabolism of AA are major contributors in the damage caused by GSH depletion. In addition, these data indicate that the resistance of pure neuronal cultures to BSO-induced damage is likely due to reduced availability

of free AA in the absence of glial cells. At present we are measuring the activity of PLA_2 at various times after exposure to BSO to determine whether depletion of GSH results in activation of PLA_2 and the time course of this change.

2c. Is the release of arachidonic acid increased during GSH depletion?

To examine that we treated mesencephalic cultures with [^{3}H]AA for 24 h to label membrane phospholipids. Following incubation, the [^{3}H]AA was removed by several washes and the cultures were treated with BSO to deplete GSH. Our data (Appendix, Fig. 5) show that [^{3}H]AA release was higher in the BSO treated cultures before any damage could be detected by the MTT assay. These results, taken together with the previous data, suggest that the release of AA precedes damage to the cell membranes and is likely to participate in the events that lead to cell death.

2d. Toxicity of arachidonic acid metabolites, via the LOX pathway, to pure neuronal and mixed cultures.

Our previous studies (Kramer et al., in Appendix) suggest that superoxide anion, generated during the metabolism of AA by LOX, plays a very important role in the toxicity of GSH depletion. The major metabolic products of AA metabolism by 12-LOX, the brain isoform involved in GSH depletion-induced toxicity (Li *et al.*, 1997), include hydroperoxyeicosatetraenoic acid (HPETE) and hydroxyeicosatetraenoic acid (HETE). Fatty acid radicals are intermediates formed during AA metabolism and highly reactive oxygen radicals are produced when HPETE is

converted to HETE (Katsuki and Okuda, 1995). To determine whether oxygen free radicals produced during the conversion of HPETE to HETE we treated mixed and pure neuronal mesencephalic cultures with 2 and 5 μM HPETE and HETE in the presence or absence of 50 μM BSO. Incubation with HPETE caused some damage to the neurons with normal GSH content, particularly at the higher concentration (Appendix Fig. 6). It is interesting that pure neuronal cultures were slightly more sensitive to HPETE than the cultures containing glial cells. When GSH levels were reduced by BSO, HPETE became extremely toxic. HETE caused no damage to the cultures (Appendix Fig. 6) under normal or reduced GSH levels.

These experiments support further our hypothesis that depletion of GSH results in increased release of AA, which when metabolized by LOX produces oxygen free radicals, including superoxide. The loss of GSH compromises the antioxidant capabilities of the cells and does not allow them to withstand the additional oxidative stress caused by AA metabolism, resulting in cell death.

A manuscript describing the above data is presently in preparation.

KEY RESEARCH ACCOMPLISHMENTS:

Our research demonstrated the following facts relating to the cause of degeneration of brain cells depleted of glutathione:

- Glutathione depletion induces increased release of arachidonic acid in mesencephalic cultures
- Inhibition of phospholipase A_2, which prevents arachidonic acid release, protects cells from damage caused by glutathione depletion

- Exposure to arachidonic acid causes cell death to GSH depleted cells
- Inhibition of arachidonic acid metabolism by lipoxygenation protects cells from damage caused by glutathione depletion and/or exposure to arachidonic acid
- Oxygen free radical-producing metabolites of lipoxygenase are damaging to glutathione depleted cells
- Up-regulation of superoxide dismutase protects cells from damage caused by glutathione depletion

REPORTABLE OUTCOMES:

Manuscript:. Kramer, BC, Yabut, JA, Robakis, T and Mytilineou, C. (2001) Lipopolysaccharide up-regulates manganese superoxide dismutase in mesencephalic cultures and protects from cell death caused by glutathione depletion. *Submitted for publication*

Abstracts and Presentations

Society of Neuroscience, November 2000, New Orleans: Mytilineou, C., Kramer, B.C. and . Yabut, J.A.. Arachidonic acid release and toxicity in glutathione (GSH) depleted mesencephalic cultures.

Oxygen Radical Society, April 2001, Maui: Kramer, B.C., Yabut, J.A. and Mytilineou, C. Up-regulation of Mn-SOD by lipopolysaccharide protects mesencephalic cultures from glutathione depletion.

Society of Neuroscience, November 2001, San Diego: Yabut, J.A., Kramer, B.C, and Mytilineou, C. Lipopolysaccharide treatment protects mesencephalic cultures from glutathione depletion.

CONCLUSIONS:

Parkinson's disease is characterized by a decrease in the levels of GSH in the substantia nigra. The goal of our research is to understand the events that lead to neuronal degeneration when there is depletion of GSH. The research during the second year of the grant period provided some important information towards this goal. We were able to demonstrate that when glutathione levels are reduced in mesencephalic cultures there is an increased release of arachidonic acid, which is metabolized by lipoxygenase producing toxic oxygen free radicals. Superoxide is one of the free radicals responsible for the damage caused by GSH depletion and increased availability of superoxide dismutase can prevent the damage caused by GSH depletion in mesencephalic cultures. This information is very important when addressing ways to prevent the progressive degeneration of dopamine neurons in Parkinson's disease.

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Li Y, Maher P, Schubert D. A role for 12-lipoxygenase in nerve cell death caused by glutathione depletion. Neuron 1997; 19: 453-63.

Moore SA, Yoder E, Murphy S, Dutton GR, Spector AA. Astrocytes, not neurons, produce docosahexaenoic acid (22:6 omega-3) and arachidonic acid (20:4 omega-6). J Neurochem 1991; 56: 518-24.

Mytilineou C, Kokotos Leonardi ET, Kramer BC, Jamindar T, Olanow CW. Glial cells mediate toxicity in glutathione-depleted mesencephalic cultures. J Neurochem 1999; 73: 112-9.

APPENDICES:

1. **Legends to Figures**
2. **APPENDIX - Figure 1-**
3. **APPENDIX - Figure 2**
4. **APPENDIX - Figure 3**
5. **APPENDIX - Figure 4**
6. **APPENDIX - Figure 5**
7. **APPENDIX - Figure 6**
8. **Manuscript**

LEGENDS TO FIGURES IN APPENDIX:

Figure 1. ***Time course of the protective effect of PLA_2 inhibition on the toxicity of BSO.*** Cultures were treated with the PLA_2 inhibitors arachidonyl trifluoromethyl keton (ATK; 10 μM) and methyl arachidonyl fluorophosphonate (MAFP; 10 μM) at the same time with BSO or 24 and 30 hr following BSO exposure. Cell viability was determined at 48 h after BSO with the MTT assay. PLA_2 inhibitors protected from toxicity at all time points. ***$p<0.001$ compared to BSO group. ANOVA followed by Tukey-Kramer multiple comparison test.

Figure 2. ***Time course of the protection of LOX inhibitors on BSO toxicity***. Cultures were treated with 5 μM baicalein and 10 μM NDGA 24 h prior to BSO exposurM NDGA 24 h prior to BSO expos

determined by measuring LDH release into the medium. Lipoxygenase inhibitors protected from toxicity at all time points. ***$p<0.001$ compared to BSO group. ANOVA followed by Tukey-Kramer multiple comparison test.

Figure 3. ***Toxicity of arachidonic acid in BSO treated mixed mesencephalic cultures. Protection by lipoxygenase inhibitors.*** Control and BSO treated (50 and 100 μM) mesencephalic cultures (A) were exposed to 10 μM arachidonic acid and cell viability was measured after 48 h with the MTT assay. B and C: Cell viability in cultures treated as in (A) but in the presence of 10 μM NDGA (B) or 5 μM Baicalein. a: $p<0.001$ compared to corresponding non-BSO group; b:

$p<0.001$ compared to the corresponding group not exposed to arachidonic acid. ANOVA followed by Tukey-Kramer multiple comparison test.

Figure 4. ***Toxicity of arachidonic acid in BSO treated pure neuronal mesencephalic cultures. Effect of lipoxygenase inhibition.*** Control and BSO treated cultures were exposed to 10 μM arachidonic acid. Cell viability was determine 48 h later with the MTT assay. ***$p<0.001$ compared to corresponding group not treated with BSO. ###$p<0.001$ compared to BSO + arachidonic acid not treated with NDGA. ANOVA followed by Tukey-Kramer multiple comparison test

Figure 5. ***Effect of 24 h BSO treatment on the spontaneous release of [^{3}H]arachidonic acid.*** Cultures were incubated with 0.5 μCi/ml [^{3}H]arachidonic acid for 24 h and then exposed to 50 μM BSO for another 24 h. The spontaneous release of arachidonic acid was measured for 15 and 30 min. Cell viability was determined with the MTT assay (inset) at the end of the release experiment. As expected there was no damage to the cells after 24 h exposure to BSO. *$p<0.05$; ***$p<0.001$. ANOVA followed by Tukey-Kramer multiple comparison test.

Figure 6. ***The effect of arachidonic acid metabolites on the survival of normal and glutathione depleted mesencephalic cultures.*** Cultures were treated with 50 μM BSO for 24 h and then exposed to 2 or 5 μM HPETE and HETE. Cell survival was determined after 48 h with the MTT assay. **$p<0.01$; ***$p<0.001$ compared to the corresponding non-BSO treated group.

###p<0.001 compared to untreated control. ANOVA followed by Tukey-Kramer multiple comparison test.

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July 27, 2001

U.S. Army Medical Research and Materiel Command
(MCMR-RMI-S)
504 Scott Street
Fort Detrick, MD 21702-5012

Dear Sirs,

Enclosed please find the original and 2 copies of the annual progress report for the grant award number DAMD17-99-1-9557, which covers the period from July 1, 2000 to June 30, 2001. An Appendix with graphs and a manuscript submitted for publication is also included.

We feel that we have made significant progress in our research goals and that our findings can help to understand the events that cause degeneration of dopamine neurons in Parkinson's disease and possibly provide directions for an effective treatment.

We appreciate the support of the Army, which has made this research possible.

Sincerely,

Catherine Mytilineou

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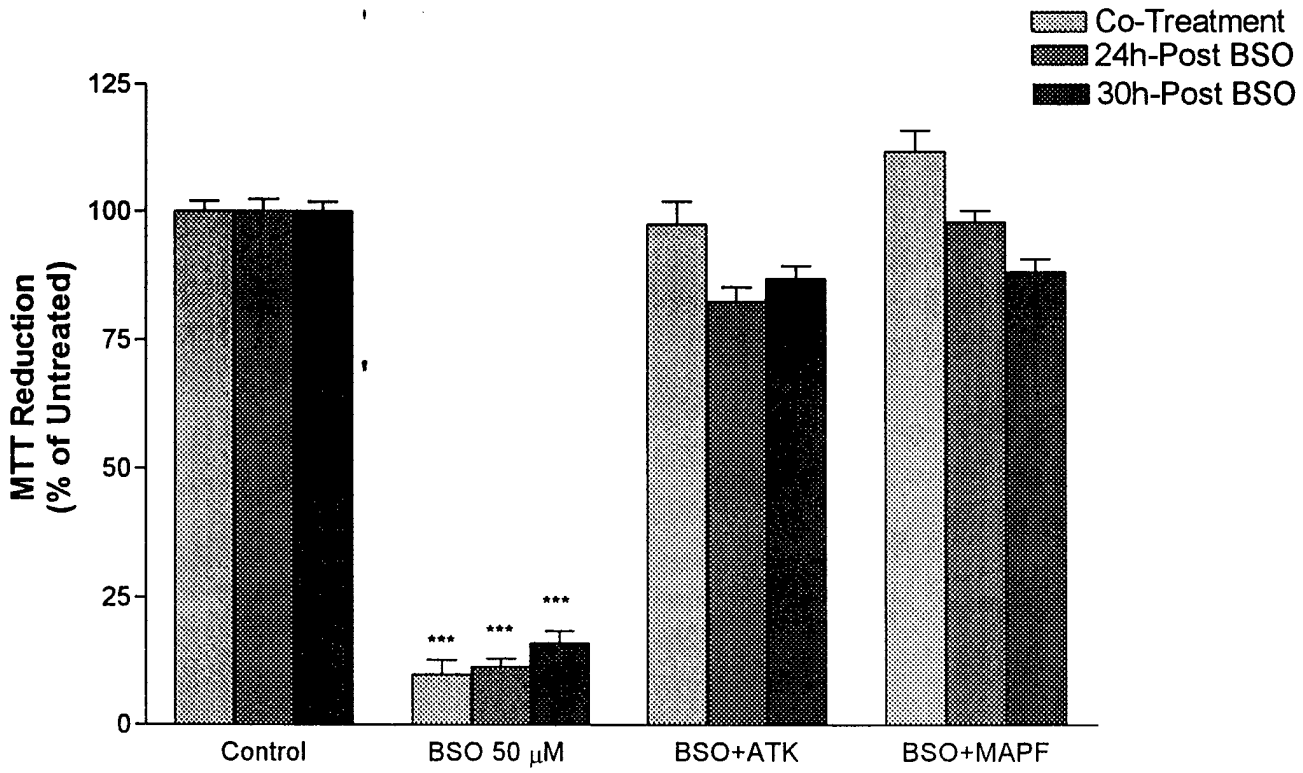
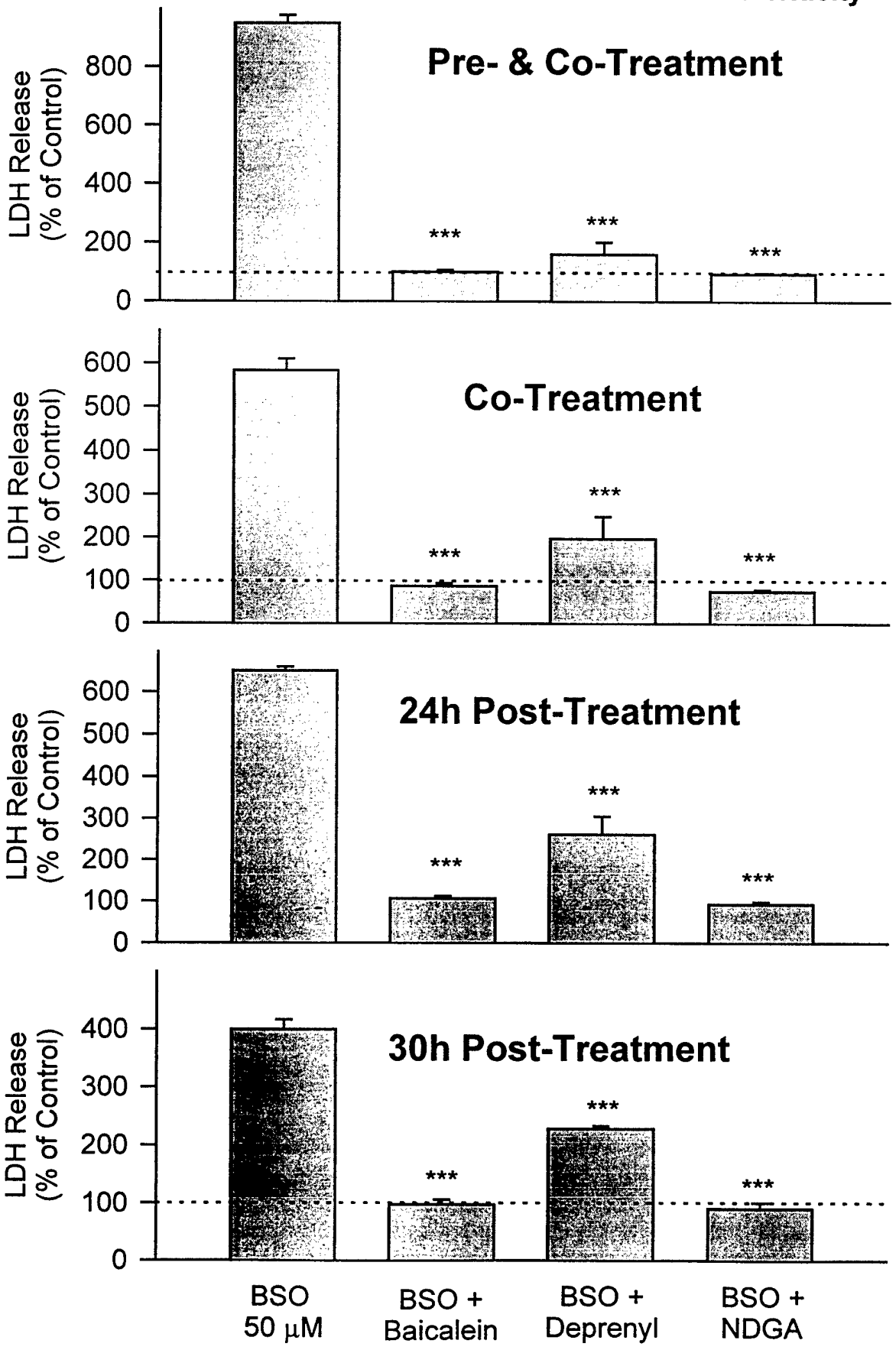
APPENDIX - Figure 1

Time course of the protective effect of PLA2 inhibition on the toxicity of BSO

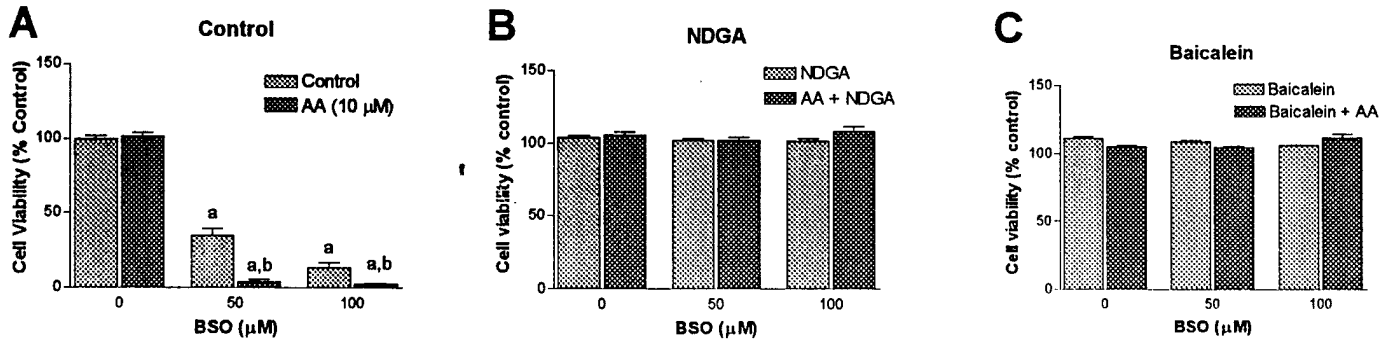


APPENDIX - Figure 2 Time course of the protection of LOX inhibitors from BSO toxicity



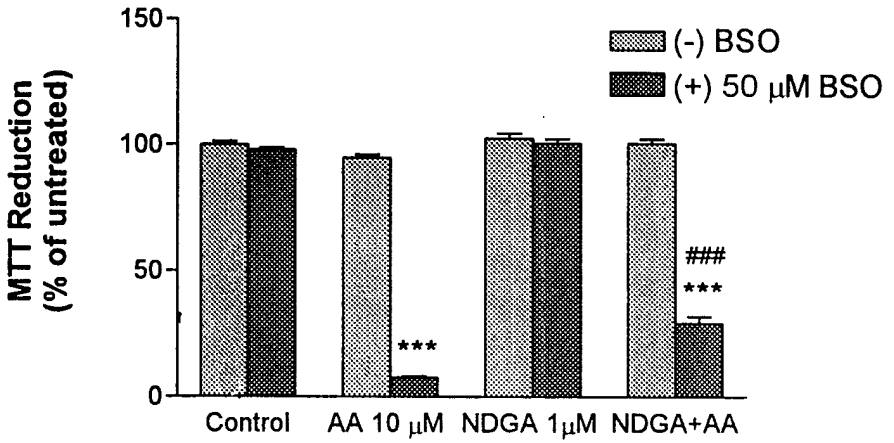
APPENDIX - Figure 3

Toxicity of arachidonic acid in BSO treated mixed mesencephalic cultures. Protection by lipoxygenase inhibitors



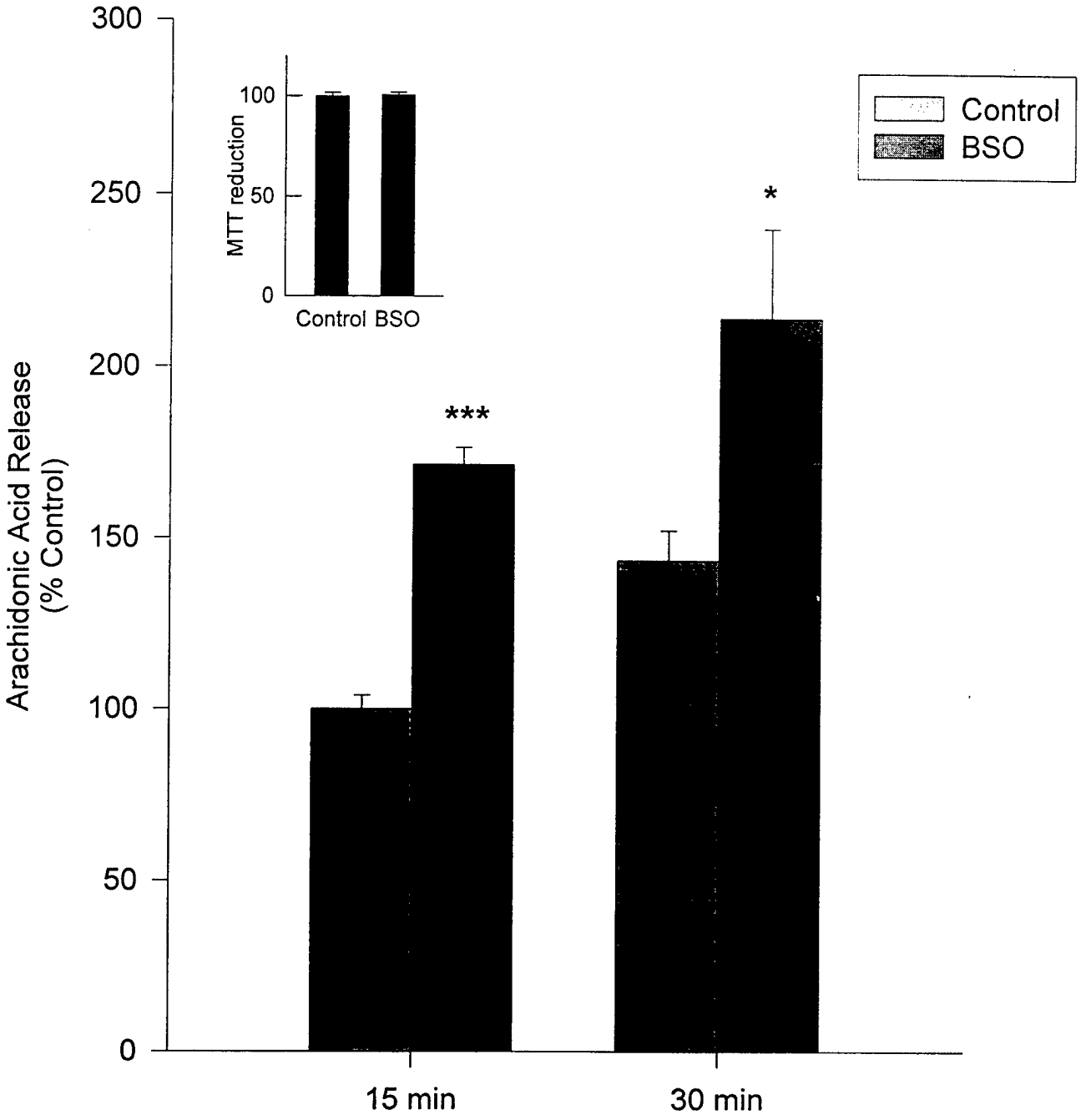
APPENDIX - Figure 4

Toxicity of arachidonic acid in BSO treated pure neuronal mesencephalic cultures. Effect of lipoxygenase inhibition.



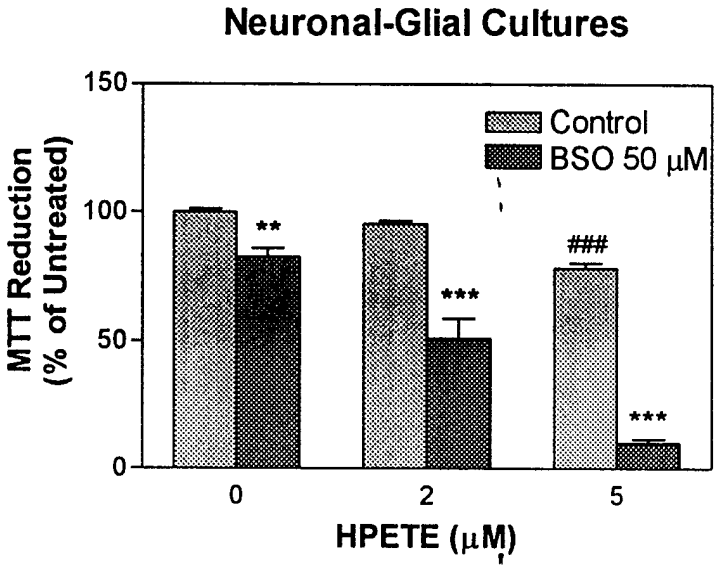
APPENDIX - Figure 5

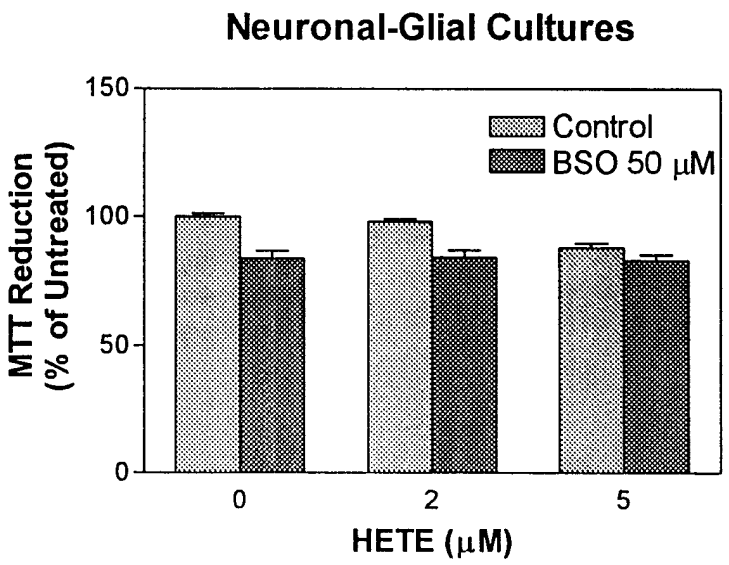
Effect of 24 h BSO treatment on the spontaneous release of arachidonic acid

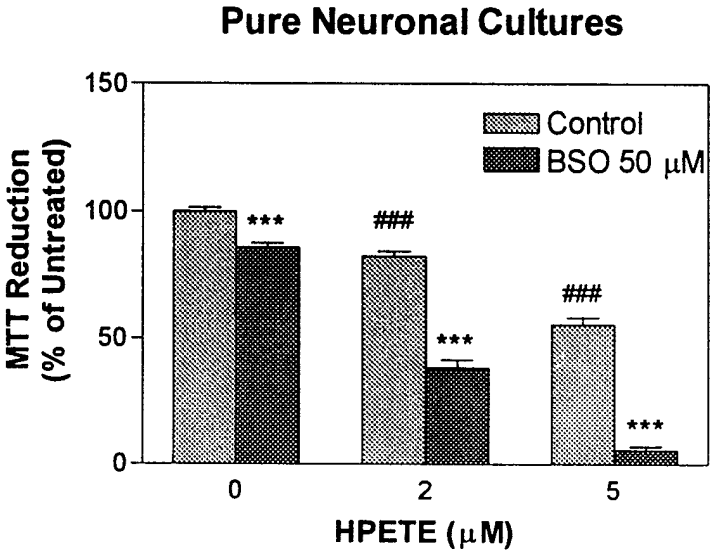


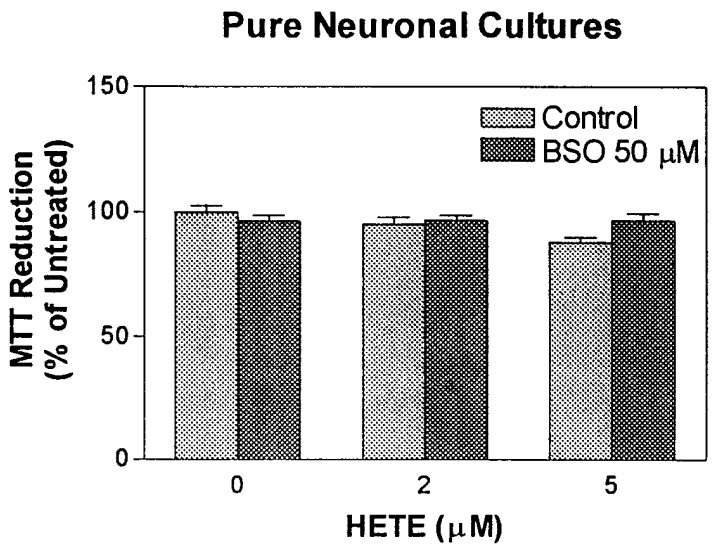
APPENDIX - Figure 6

The effect of arachidonic acid metabolites on the survival of normal and glutathione depleted mixed and pure neuronal cultures









Cellular/Molecular Neuroscience; Senior Editor: Dr. Gail Mandel

Lipopolysaccharide Up-regulates Manganese Superoxide Dismutase in Mesencephalic Cultures and Protects from Cell Death Caused by Glutathione Depletion

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Abbreviated Title: Up-regulation of MnSOD protects from GSH depletion

Number of text pages: 31

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ABSTRACT

Glutathione (GSH) is an important cellular antioxidant present at high concentrations in the brain. We previously demonstrated that depletion of GSH in mesencephalic cultures results in cell death, which can be prevented by inhibition of lipoxygenase activity, suggesting a role for arachidonic acid metabolism in the toxic events. We also showed that the presence of glial cells in the cultures contributes to the toxicity of GSH depletion. To study the role of glia on the toxicity caused by GSH depletion, we treated mesencephalic cultures with the GSH synthesis inhibitor L-buthionine sulfoximine (BSO) and the endotoxin lipopolysaccharide (LPS). LPS causes glial activation, which can increase the secretion of cytokines and the release of arachidonic acid. Our data show that LPS activated glial cells in mesencephalic cultures, increased interleukin-1β in microglia and caused depletion of GSH. However, the overall effect was protection from damage caused by treatment with BSO. The protection by LPS was not likely due to cytokines or growth factors secreted by activated glia. LPS, however, caused significant increases in the protein content and activity of Mn-dependent superoxide dismutase (SOD), suggesting a possible role for this mitochondrial antioxidant enzyme in the protective effect of LPS. This was supported by the protective effect of exogenous SOD against BSO-induced damage. Our data suggest that superoxide is the major toxic radical responsible for damage caused by GSH depletion and that up-regulation of SOD may offer protection in diseases associated with oxidative stress.

Key words: Glutathione; Lipopolysaccharide; Mesencephalic cultures; Superoxide dismutase; Oxidative stress; Parkinson's disease

Oxidative stress is believed to contribute to the degeneration of dopamine neurons in Parkinson's disease (PD). Hydrogen peroxide (H_2O_2), a major oxidative species produced normally within neurons during respiration, is also formed during the metabolism of dopamine by monoamine oxidase. The high-energy requirements coupled with the metabolism of dopamine could result in concentrations of H_2O_2 sufficient to cause oxidative stress in dopamine neurons (Cohen and Kesler, 1999). Glutathione (GSH), a major soluble antioxidant in brain, detoxifies H_2O_2 and lipid hydroperoxides (Meister, 1991). GSH is depleted in the substantia nigra in PD (Perry et al., 1982; Sofic et al., 1992) and it has been suggested that this loss may be an early event in its pathogenesis (Dexter et al., 1994).

Inhibition of γ-glutamylcysteine synthetase, the rate limiting enzyme in GSH synthesis, by L-buthionine sulfoximine (BSO) causes cell death in primary neuronal cultures and cell lines (Li et al., 1997; Mytilineou et al., 1999; Wullner et al., 1999). Inhibition of lipoxygenase activity prevents cell death, implicating arachidonic acid metabolism in the toxicity of GSH depletion (Li et al., 1997; Mytilineou et al., 1999). We have recently shown that damage from GSH depletion becomes greater in mesencephalic cultures with increased glia to neuron ratio (Mytilineou et al., 1999). The contribution of glia to the toxicity of GSH depletion is not well understood and, in view of the well-known neuroprotective role of astrocytes (O'Malley et al., 1994; Takeshima et al., 1994; Desagher et al., 1996; Wilson, 1997), such an effect may appear counter-intuitive. Several findings, however, could implicate astrocytes in arachidonic acid-mediated toxicity: (1) Within the brain, arachidonic acid is synthesized exclusively in astrocytes (Katsuki and Okuda, 1995). (2) Neurons depend on astrocytes for arachidonic acid transport, because they cannot perform the fatty acid desaturation steps necessary for its synthesis (Moore et al., 1991; Katsuki and Okuda, 1995). (3) The cytosolic form of phospholipase A_2 (PLA_2), the enzyme primarily

responsible for the release of arachidonic acid in brain, is present mainly within astrocytes (Stephenson et al., 1994).

Activation of glial cells causes secretion of cytokines, increases PLA_2 activity (Oka and Arita, 1991) and induces release of arachidonic acid in astrocytes (Stella et al., 1997; Minghetti and Levi, 1998). Theoretically, glial activation combined with GSH depletion, which also increases lipoxygenase activity (Shornick and Holtzman, 1993; Li et al., 1997), should result in excess superoxide (O_2^-) generation from the metabolism of arachidonic acid and create additional oxidative challenge to GSH depleted cells.

To study the role of glial activation in the toxicity of GSH depletion, we exposed mesencephalic cultures to a bacterial endotoxin (lipopolysaccharide; LPS), prior to treatment with BSO. We show that LPS activated microglia and astrocytes, reduced the levels of GSH and increased the cytokine IL-1β, which is toxic to GSH depleted cells. However, the overall effect of LPS was a significant protection from the toxicity of GSH depletion. Our data suggest that protection by LPS is likely due to up-regulation of Mn-dependent superoxide dismutase (SOD), the mitochondrial enzyme responsible for detoxification of O_2^-.

MATERIALS AND METHODS

Materials: Pregnant Sprague-Dawley rats were obtained from Taconic Farms (Germantown, N.Y.). MEM was purchased from GIBCO-Life Technologies (Grand Island, N.Y.), horse serum from Gemini (Calabasas, CA) and NU® serum from Collaborative Biomedical Products (Bedford, MA). LPS (from *Escherichia coli* serotype 026:B6) and other chemicals were obtained from Sigma (St. Louis, MO). Monoclonal antibodies to glial fibrillary acidic protein (GFAP) were purchased from Sigma (St. Louis, MO) and to tyrosine hydroxylase (TH) from Boehringer

Mannheim (Indianaposis, IN). Monoclonal OX-42 antibodies against the rat microglial surface antigen complement receptor type 3 (Mac-1) (Perry et al., 1985) were purchased from Chemicon (Temecula, CA). Polyclonal antibodies to IL-1β were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), to Cu/ZnSOD from Chemicon and to MnSOD from StressGen Biotechnologies (Victoria, BC, Canada).

Cell Cultures: The protocols for handling animals and preparing cell cultures followed the NIH guidelines and were approved by the institutional review committee. Mesencephalic cultures were prepared from embryonic rats on the 14th day of gestation as described previously (Mytilineou et al., 1999). In brief, the mesencephalon was dissected free of meninges and collected in Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (PBS). The tissue was mechanically dissociated into a single cell suspension and plated in 24 or 6 well plates pre-coated with L-polyornithine (0.1 mg/ml) at a density of 300,000 cells/cm^2. The medium consisted of MEM supplemented with 2 mM glutamine, 33 mM glucose, 10% horse serum and 10% NU® serum. Treatment began on the 5-6th day *in vitro,* at which time the medium was changed to MEM containing only 5% NU® serum. The method of McCarthy and de Vellis (1980) was used to prepare purified astrocytes. Briefly, cortical cultures prepared from newborn rats were plated in 75 cm^2 flasks at 5X10^6 cells/flask. After 7 days *in vitro* the flasks were shaken overnight on a rotary shaker at 250 rpm and the following day the floating cells were removed and the remaining attached astrocytes dislodged with Versene® and plated in 24 well plates.

Immunocytochemistry: Cells were plated on polyornithine coated glass coverslips in 24 well plates. They were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature and permeabilized and blocked with 0.3% Triton X-100 ad 3% bovine serum albumin (BSA) for 30

min. Primary antibodies used were: anti-GFAP (1:1000), OX-42 (1:250), anti-microtubule associated protein (MAP-2 1:500), anti-IL-1β (1:250) and anti-TH (1:1000). Cultures were exposed to the primary antibodies overnight at 4°C. Secondary antibodies conjugated to Alexa fluorescent dyes (Molecular Probes; Eugene, OR) were used at a dilution of 1:1000 for 30 min. The cultures were observed with an Olympus fluorescence microscope and the images recorded with a Spot video camera.

[^{3}H]Dopamine uptake: Measurement of dopamine uptake was performed as described previously (Mytilineou et al., 1998). Cultures were rinsed with Kreb's phosphate buffer (pH 7.4) and incubated for 30 min at 37°C with the same buffer containing 0.2 mg/ml ascorbic acid and 0.5 μCi/ml [^{3}H]dopamine (32.6 Ci/mmol; NEN, Boston MA). After rinsing, the radioactivity was extracted with 1 ml 95% ethanol, which was added to vials containing scintillation cocktail and the radioactivity measured in a scintillation spectrometer (Packard Tri-Carb 2100). Cultures treated with the neuronal dopamine uptake blocker mazindol (10 μM) were used as blanks.

Cell Viability Assays - MTT Assay: Cell viability was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay, as described previously (Han et al., 1996). In brief, 50 μl of a 5 mg/ml solution of MTT was added to each cell culture well containing 0.5 ml medium. After a 3 h incubation at 37°C, the medium was carefully removed and the formazan crystals were dissolved in 1 ml isopropyl alcohol by gentle shaking of the plate. Absorbance was determined at 570 nm in a microplate reader (Spectramax 250, Molecular Devices Corporation, Sunnyvale CA).

LDH assay: A modification of the method by Bergmeyer et al. (1963) was used to determine LDH activity in the culture medium and the cells. Medium was collected, centrifuged to remove

debris and frozen at -80°C until assay. Cells were freeze thawed (x 3) in 1.0 ml feeding medium, the medium was collected, centrifuged and the supernatant frozen at -80°C. 50 µl of supernatant and 100 µl of NADH (1.2 mg/ml H_2O stock) were added to 850 µl of buffer and the samples were vortex-mixed. 50 µl of feeding medium was used for blanks. Triplicate aliquots (250 µl) were placed into 96-well plates at room temperature and reaction was initiated by addition of 25 µl of sodium pyruvate (0.36 mg/ml H_2O stock). The rate of disappearance of NADH was measured at 340 nm using a plate reader.

Glutathione assay: GSH was quantified using a modification of a standard recycling assay based on the reduction of 5,5-dithiobis-(2-nitrobenzoic acid) with glutathione reductase and NADPH (Tietze, 1969). In brief, the medium was carefully aspirated from the culture wells, 300 µl of 0.4 N PCA was added and the plates were kept on ice for 30 min. The PCA was then collected and stored at –70°C until assayed. Both oxidized (GSSG) and reduced (GSH) forms of glutathione are measured with this assay. However, because of the small amounts of GSSG present in mesencephalic cultures (~5% of total; Mytilineou et al., 1993), the values obtained were considered to represent GSH content.

The tissue attached to the bottom of the wells after removal of the PCA was dissolved in equal volumes of 20% SDS and 0.5 N NaOH and used for protein determination according to the method of Lowry (Lowry et al., 1951) with BSA as a standard.

SOD Assay: Cells plated in 6 well plates were collected in cold PBS (0.1 ml/well). 3-6 wells were pooled for each sample. Cells were sonicated on ice, centrifuged at 4,000 x g for 10 min at 4°C and dialyzed overnight in PBS at 4°C (Slide-A-Lyzer dialysis Cassettes, 10K cut-off;

PIERCE, Rockford, IL). SOD activity was assayed in 50 µl of the dialysate according to the assay developed by McCord and Fridovich (1969), modified for use with a microplate reader. In brief, 100 µl of xanthine solution (4 mM in 0.01 M NaOH) and 10 µl of cytochrome c (partially acetylated; 0.5 mg/ml) were added to 900 µl of 50 mM phosphate buffer. After the addition of 50 µl of sample, buffer for blanks or SOD for standards, triplicates of 250 µl were placed in the wells of a 96 well plate and the reaction was initiated by the addition of 25 µl/well xanthine oxidase (0.75 U/ml in 0.1 mM EDTA). The reduction rate of cytochrome c by superoxide radicals was monitored at 550 nm at 25°C for 10 min. Total SOD activity in the samples was determined from a standard curve and was corrected for protein content. MnSOD activity was determined after inhibition of the Cu/ZnSOD by a 5 min incubation with 2 mM KCN.

Sodium docedyl sulfate-polyacrylamide gel eletrophoresis (SDS-PAGE): The dialyzed cell homogenates used for SOD assays were also used for Western blotting. Samples containing 20 µg of protein were mixed with 20 µl laemmli buffer. The proteins were resolved on a 12% SDS-PAGE (Bio –RAD, Hercules, CA) and transferred to Hybond^{TM}-P PVDF membrane (Amersham Pharmacia, Piscataway, NJ) for 1 hour at 15V, using a Trans-Blot Semi-Dry Transfer Cell (Bio – RAD, Hercules, CA). Membranes were incubated in blocking solution (5% milk, 5% newborn-calf serum) in TTBS (20mM Tris-HCl, 500mM NaCl, 0.1% Tween-20, pH 7.6) at 4°C overnight and then with polyclonal antibodies to Cu/ZnSOD or Mn/SOD and with monoclonal antibody to TH in blocking solution for 2 hr. They were then washed with TTBS and incubated with horseradish-peroxidase conjugated goat anti-rabbit IgG (whole molecule) antibody (ICN, Aurora, OH) in blocking solution for 1 hour at room temperature. After washing with TTBS the

membranes were visualized with ECL Plus Western blot detection system (Amersham Pharmacia, Piscataway, NJ).

RESULTS

LPS causes glial activation in mesencephalic cultures.

LPS, an endotoxin used extensively to induce glial activation *in vivo* (Castano et al., 1998) and *in vitro* (Bronstein et al., 1995; Jeohn et al., 1998), was added to the medium of mesencephalic cultures at day 5 *in vitro*. Specific markers for astrocytes and microglia were used to test for glial activation. After 72 h exposure to 10 μg/ml LPS, immunocytochemistry with antibodies to GFAP revealed an increase in the number and processes of astrocytes compared with the controls (Fig. 1A and B). The number and size of microglia cells, labeled with OX-42 was also increased after LPS treatment (Fig. 1C and D). We also tested for the presence of the pro-inflammatory cytokines interleukin-1β (IL-1β) and TNF-α, which are secreted by activated glial cells (Benveniste, 1998). IL-1β was present exclusively in OX-42 labeled cells, indicating microglial localization of this cytokine (Fig. 1E, F). Labeling for IL-1β was weak in most microglia of control cultures (Fig. 1E). An increase in IL-1β immunoreactivity in Mac-1 positive cells was observed after LPS treatment (Fig. 1F). Antibodies to TNF-α labeled both neurons and astrocytes and there was no apparent difference in intensity or distribution between control and LPS treated cultures (not shown).

LPS provides protection against BSO-induced toxicity.

To examine the effect of glial activation on GSH depletion-induced damage, cultures were pretreated with 10 μg/ml LPS for 24 h and then exposed to the GSH synthesis inhibitor,

BSO, for an additional 48 hr. LPS was present in the medium during BSO treatment. In agreement with our previous studies (Mytilineou et al., 1998; Mytilineou et al., 1999), BSO at 10 and 50 μM caused significant damage, as assessed by the MTT assay (Fig. 2A). Treatment with LPS significantly attenuated the BSO-induced damage (Fig. 2A). Cell viability was reduced to 42% and 9% of control values after a 48 h treatment with 10 and 50 μM BSO respectively, and LPS treatment improved survival to 65% and 56% of control with the same BSO treatment.

LPS concentrations ranging from 1 to 50 μg/ml were equally effective in protecting from BSO toxicity (Fig. 2B). In the experiment presented in Fig. 2B, 50 μM BSO caused only moderate loss of cell viability after 48 h (47% loss) and LPS prevented this damage at all concentrations tested. Differences in the extent of toxicity caused by BSO in some experiments, is believed to be due to variations among primary cultures from different preparations.

It has been reported that LPS causes neuronal toxicity *in vitro*, which can be selective for dopamine neurons (Bronstein et al., 1995; Jeohn et al., 1998), and is sometimes achieved with ng/ml concentrations of LPS (Liu et al., 2000). To address the apparent conflict between these reports and our data, we compared the effect of LPS from *Escherichia coli*, serotype 0111:B4 used by Liu *et al* (2000), with the serotype 026:B6 used in our study, at concentrations ranging from 0.1ng/ml to 10 μg/ml. Examination of live cells with phase contrast microscopy showed no detectable toxicity in our culture system at any LPS concentration. Measurement of [^{3}H]dopamine uptake, used in the study by Liu *et al.* (2000) to assess for selective damage to dopamine neurons, also demonstrated no toxicity of LPS (Table 1). We speculate that the lack of LPS-induced damage in our experiments was likely due to the different cell culture conditions (feeding medium, cell density, etc.).

LPS lowers GSH levels in astrocytes and mesencephalic cultures.

We examined whether protection by LPS was the result of a direct effect on GSH levels. Since astrocytes, the major glial component in mesencephalic cultures, are important regulators of neuronal GSH (Sagara et al., 1993), we also tested the effect of LPS on GSH levels in cultures enriched in astrocytes. Astrocytes were treated with 1 or 10 μg/ml LPS for 24 h and then exposed to 10 μM BSO for an additional 48 h. After BSO treatment alone the levels of GSH were reduced to 43% of control values (Fig. 3A). In cultures treated with 1 or 10 μg/ml LPS, BSO caused an even greater decrease in GSH, to 14% and 13% of control levels respectively. Treatment with LPS alone caused significant reduction in GSH levels as well (to 73% and 76% of control with 1 and 10 μg/ml LPS, respectively). Neither LPS nor BSO treatment caused any loss in the viability of astrocytes as determined with the MTT assay (results not shown).

Treatment of mixed neuronal-glial cultures with LPS also caused a significant loss of GSH, reducing the levels to 77% and 25% of control values with 1 and 10 μg/ml, respectively (Fig. 3B). Treatment with 10 μM BSO for 48 h caused severe loss of cells in cultures not exposed to LPS and the GSH levels were only about 2% of controls. However, the GSH content in the few surviving cells may actually be underestimated when expressed in nmol/per mg protein, as protein measurement includes cell debris still attached to the culture dish. GSH levels in cultures treated with 10 μM BSO and 1 μg/ml LPS were 15% of control values, while 10 μg/ml LPS decreased GSH to levels not detectable by our assay, even though no detectable damage to the cells could be observed.

Possible mechanisms of LPS protection

Secretion of growth factors and cytokines

Activated glial cells secrete both toxic and trophic substances. We tested the effects of insulin-like growth factor I (IGF-I), transforming growth factor β (TGF-β) and basic fibroblast growth factor (bFGF), known to be secreted by glial cells, on the toxicity of BSO in mesencephalic cultures (Fig. 4). In these experiments BSO-induced damage was determined by measuring the amount of LDH released into the culture medium. The concentration of growth factors used was 50 or 100 ng/ml and was selected for maximal trophic activity towards neurons (Bouvier and Mytilineou, 1995). bFGF protected mesencephalic cells from toxicity at the lower concentrations of BSO (5 and 10 μM; Fig. 4A). With 50 μM BSO the damage was extensive (64% of total LDH was released into the medium) and bFGF was unable to confer any protection. Neither TGF-β nor IGF-I had any effect on BSO toxicity (Fig. 4B and 4C). Similar results were obtained when these growth factors were used at 100 ng/ml.

A number of pro- and anti-inflammatory cytokines are secreted upon activation of glial cells (Giulian et al., 1993; Aschner, 1998; Minghetti and Levi, 1998). We tested whether the pro-inflammatory cytokines IL-1β, interferon-γ (INF-γ) or tumor necrosis factor-α (TNF-α) can modify BSO-induced toxicity. As shown in Fig. 5, exposure to IL-1β significantly increased BSO toxicity, while INF-γ and TNF-α had no effect (Table 2). The cytokines IL-6 and IL-10, which can have anti-inflammatory properties and may be neuroprotective were also tested, but failed to provide protection against BSO toxicity (Table 2).

Up-regulation of antioxidant enzymes

LPS has been shown to up-regulate the antioxidant enzyme SOD in glial cultures (Del Vecchio and Shaffer, 1991; Mokuno et al., 1994). We examined the effect of LPS treatment (10 μg/ml for 48 hr) on SOD activity and protein levels in mesencephalic cultures. Treatment with LPS caused a greater than 2-fold increase in MnSOD activity, but had no significant effect on Cu/ZnSOD (Fig. 6A). MnSOD protein levels were also higher after LPS treatment (Fig. 6B), with no change in Cu/ZnSOD. To test the possibility that increased levels of SOD played a role in the protection from BSO toxicity we treated cultures with BSO in the presence or absence of SOD and catalase (Fig. 7). SOD added to the medium (200 or 500 units/ml), completely prevented BSO toxicity, while catalase (100 or 200 units/ml) provided no protection.

The localization of Cu/Zn- and MnSOD in mesencephalic cultures was examined using antibodies specific for the two enzymes. Labeling for Cu/ZnSOD was diffuse and was mostly present in neurons. No difference could be observed in Cu/ZnSOD labeling between control and LPS treated cultures (Fig. 8 A, B). Labeling for MnSOD was punctate, reflecting its mitochondrial localization. In control cultures, MnSOD was present primarily within neurons, as demonstrated by its co-localization with MAP2 (Fig. 8C). Very little labeling could be found in GFAP positive astrocytes (Fig. 8E). However, after treatment with LPS very intense labeling appeared within astrocytes (Fig. 8F). The intensity of MnSOD labeling in the neurons did not seem to be affected by LPS (Fig. 8D).

The microglia activators fMLP and Zymosan A do not modify BSO toxicity.

To determine whether other glial activators protect against the toxicity of GSH depletion, we tested the selective microglia activators formyl-methionyl-leucyl-phenylalanine (fMLP) and

zymosan A particles (North, 1978; Zietlow et al., 1999). Immunocytochemistry with GFAP and Mac-1 indicated that fMLP and zymosan A particles increased the number and size of microglial cells, without significantly modifying the appearance of astrocytes (not shown). Neither zymosan A, nor fMLP modified the toxicity of BSO significantly at any of the concentrations tested. Cell viability in cultures treated with 100 or 200 μg/ml zymosan A in the presence of BSO (50μM) was 14.8±1.8 and 10.2±1.5% of control respectively, compared to 7.7±0.3% of control when treated with BSO alone. Similarly cell viability in cultures treated with 0.01, 0.1 or 1 μM fMLP in the presence of 50 μM BSO was 16.1±1.7, 13.9±1.2 and 14.4±1.6 % of control respectively, compared to 18.8±1.5% of control in cultures treated with BSO alone. There was no effect of zymosan A or fMLP on cell viability in control cultures. These data suggest that activation of microglia does not necessarily result in protection from damage caused by GSH depletion.

DISCUSSION

Our study shows that activation of glial cells by LPS protects mesencephalic cultures from oxidative damage caused by the depletion of GSH. Glial activation was confirmed by the changes in morphology and the increase in the size of microglia (Kreutzberg, 1996) and by the increased number of GFAP-positive astrocytic processes (Aschner, 1998). The pro-inflammatory cytokine IL-1β was also increased in microglia after treatment with LPS, a further indication of an activated state (Giulian et al., 1994).

It has been reported that LPS is selectively toxic to dopamine neurons in mesencephalic cultures (Bronstein et al., 1995). Although LPS did not cause cell death or reduction in the uptake of dopamine in our culture system, it did result in oxidative stress, which was implied by the reduction in GSH content in both astrocytes and mixed neuronal cultures. LPS treatment

potentiated the effect of BSO on GSH depletion, which suggests that the inflammatory response of glial cells has the potential to cause oxidative damage. Furthermore, exposure to IL-1β increased BSO-induced damage, indicating that under conditions of oxidative stress IL-1β released by activated microglia can contribute to neurodegeneration. However, in spite of these apparently harmful effects, the overall result of LPS treatment was a significant protection from toxicity caused by depletion of GSH.

Both astrocytes and microglia have the potential to provide support of neuronal survival *in vitro* (Engele et al., 1991; O'Malley et al., 1992; Nagata et al., 1993; Takeshima et al., 1994; Hou et al., 1997). Astrocytes stimulate neuronal growth, survival and regeneration by secretion of growth factors and extracellular matrix proteins (Muller et al., 1995; Fawcett, 1997). Following activation, astrocytes secrete both pro- and anti-inflammatory cytokines and growth factors (Aschner, 1998). We examined whether activated glial cells protected from damage caused by GSH depletion through the secretion of the growth factors bFGF, IGF-I and TGF-β. In a previous study we showed that bFGF reduced the damage caused by combined 6-hydroxydopamine and BSO treatment of mesencephalic cultures (Hou et al., 1997). In the present experiments bFGF provided some protection from BSO toxicity when damage was not extensive, but it was substantially less effective than LPS. Therefore, it seems unlikely that secretion of bFGF by activated glial can by itself explain the protective effect of LPS treatment, although it may be contributory. TGF-β and IGF-I had no effect. LPS is a potent activator of IL-6 in astrocytes (Benveniste et al., 1990) and IL-6 can have both trophic and toxic effects on neurons (Gruol and Nelson, 1997). However, in our experiments IL-6 was neither toxic nor protective to GSH depleted cells. Similarly the cytokines IL-10, TNFα and INF-γ did not modify the damage caused by BSO treatment.

Support of neuronal survival by astrocytes is believed to be due in part to the scavenging of extracellular reactive oxygen species (Peuchen et al., 1997; Drukarch et al., 1998). Compared to neurons, astrocytes and microglia have higher concentrations of GSH (Sagara et al., 1993; Makar et al., 1994; Chatterjee et al., 1999; Mytilineou et al., 1999) and they are enriched in the antioxidant enzymes GSH-peroxidase, SOD and catalase (Makar et al., 1994; Desagher et al., 1996). Recently, it has been shown that priming with small doses of LPS can protect mice against ischemia (Tasaki et al., 1997; Dawson et al., 1999; Ahmed et al., 2000; Bordet et al., 2000). The beneficial effect paralleled the induction of inflammation and was attributed to a compensatory activation of SOD by LPS (Bordet et al., 2000). Exposure of neuronal and glial cell cultures to LPS also up-regulates MnSOD, the inducible form of SOD present in the mitochondria (Kifle et al., 1996; Yu et al., 1999). In our study we found that LPS caused a significant increase in both protein content and activity of MnSOD in mesencephalic cultures. The increase in protein occurred primarily in GFAP-positive astrocytes. There was no change in the Cu/ZnSOD, the constitutive form of the enzyme present in the cytoplasm. A role for SOD in the LPS-induced protection was supported by the finding that addition of SOD to mesencephalic cultures completely prevented BSO-induced damage. Although the effect of exogenous SOD was likely extracellular, the protection observed probably resulted from scavenging of excessive O_2^- generated as a consequence of GSH depletion. Addition of catalase did not offer protection from BSO toxicity, suggesting that extracellular accumulation of H_2O_2 was not the primary cause of cell death.

There are several potential sources of O_2^- formation in cells, including oxidative phosphorylation used by the mitochondria for the generation of ATP. O_2^- is also formed during the metabolism of arachidonic acid by lipoxygenase, when hydroperoxyeicosetetraenoic acid

(HPETE), the primary product of arachidonic acid metabolism, is converted to hydroxyeicosatetraenoic acid (HETE) (Katsuki and Okuda, 1995). Our results suggest the O_2^-, rather than H_2O_2, is responsible for the toxic events that follow GSH depletion. Merad-Saidoune et al. (1999) also reached a similar conclusion in a recent study showing that over-expression of SOD prevents mitochondrial damage caused by GSH depletion. Normally the concentration of O_2^- in the cells remains low as a result of the action of SOD. However, during GSH depletion SOD may not be able to handle the excess O_2^- generated from, among other sources, the metabolism of arachidonic acid by lipoxygenase. Arachidonic acid can also promote the generation of reactive oxygen species by directly inhibiting the mitochondrial respiratory chain (Cocco et al., 1999). When O_2^- levels become high, nitric oxide (NO), an otherwise non-toxic free radical, competes with SOD and combines rapidly with O_2^- to form peroxynitirte ($ONOO^-$). The damage to mitochondria caused by GSH depletion in neuronal and glial cultures has been shown to be the result of peroxynitrite production (Bolanos et al., 1995). Our earlier studies showing that inhibition of nitric oxide synthase offers some protection from BSO toxicity in mesencephalic cultures, also suggest that NO may play some role on the damage caused by GSH depletion (Mytilineou et al., 1999). Peroxinitrite can damage cells because it reacts slowly with proteins acting as a selective oxidant and nitration agent (Bartosz, 1996). Nitrotyrosine is the product of the reaction of peroxinitrite with tyrosine and tyrosine residues (Reiter et al., 2000). The presence of nitrotyrosine in post-mortem tissues in Parkinson's disease (Good et al., 1998) and other neurodegenerative disorders (Abe et al., 1995; Good et al., 1996; Sasaki et al., 2000), indicates that increased concentrations of O_2^- may contribute to their pathogenesis. Our data suggest that up-regulation of SOD activity may provide protection from oxidative stress and delay the progress of neurodegenerative diseases.

Our study also illustrates the dependence of neurons on the surrounding glial cells. During conditions of oxidative stress, such as GSH depletion, the state of the surrounding glia can determine whether neurons will survive or die. Understanding of the relationships between neurons and glia should provide further insight into the process of neuronal degeneration, which contributes to the progression of neurological disorders.

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Table 1. The effect of LPS obtained from different *E. coli* clones on [^{3}H]dopamine uptake in mesencephalic cultures.

LPS concentration (ng/ml)	[^{3}H]Dopamine Uptake (% of control)	
	Clone 0111:B4	Clone 026:B6
0	100. 0 ± 3.8	100.0 ± 1.7
0.1	91.3 ± 1.8	99.2 ± 3.9
1	99.2 ± 3.4	97.8 ± 1.9
10	97.2 ± 1.4	93.8 ± 6.7
100	96.3 ± 4.4	93.1 ± 2.5
10,000	89.0 ± 1.4	98.0 ± 6.7

Mesencephalic cultures were exposed on the 5th day *in vitro* to LPS for 24 h and then treated again with LPS for an additional 48 h in order to duplicate the conditions used in the experiments examining the effect of LPS on the damage caused by GSH depletion. [^{3}H]Dopamine uptake was performed at the end of LPS treatment. Analysis of variance showed no significant differences in uptake following LPS treatment.

Table 2. The cytokines TNF-α, INF-γ, IL-6 and IL-10 do not modify the toxic effect of GSH depletion.

	LDH Release (% of Total)			
Cytokine (ng/ml)	**Control**	**BSO**	**Cytokine**	**Cytokine + BSO**
TNF-α (20)	27.8 ± 1.9	61.1 ± 3.3	27.7 ± 2.7	50.9 ± 1.9
INF-γ (50)	12.3 ± 0.4	60.6 ± 1.5	13.2 ± 0.6	60.1 ± 1.2
INF-γ (100)			11.5 ± 0.3	61.2 ± 1.1
IL-6 (50)	7.4 ± 0.2	65.0 ± 2.9	7.4 ± 0.3	62.2 ± 2.7
IL-6 (100)			7.4 ± 0.6	69.0 ± 1.7
IL-10 (50)	7.8 ± 0.3	60.6 ± 3.8	7.4 ± 0.2	58.6 ± 3.5
IL-10 (100)			7.1 ± 0.5	64.7 ±1.3

Mesencephalic cultures were pre-treated on the 5th day *in vitro* with the cytokines and 24 h later exposed to 50 μM BSO for an additional 48 hr. Cytokines were present during BSO treatment. LDH release into the medium was used as an index of cell damage. The values represent the LDH activity in the medium expressed as a percentage of total LDH (medium + cells) ± SEM (N=8 from 2 experiments).

LEGENDS TO FIGURES

Figure 1. Treatment with LPS causes activation of astrocytes and microglia in mesencephalic cultures. Immunocytochemistry for GFAP (A, B), OX-42 (C, D) and IL-1β (E, F) in control cultures (A, C, E) and cultures treated with 10 μg/ml LPS for 72 h. Increased labeling of GFAP positive astrocytes was apparent in cultures treated with LPS (compare A and B). OX-42 labeling of microglia showed an increase in the size after treatment with LPS (compare C and D). The number of microglia was also increased after LPS treatment. Panels E and F show the same fields as C and D double labeled for IL-1β. In control cultures (E), some microglia (arrow) express low levels of the cytokine. After LPS treatment (F), all OX-42 labeled cells show intense immunoreactivity for IL-1β. Bar = 25 μm.

Figure 2. LPS protects mesencephalic cultures from the toxicity of BSO treatment. (A) Cultures were exposed to 10 μg/ml LPS for 24 h and then treated with 10 or 50 μM BSO for an additional 48 h in the presence or absence of LPS. (B) Cultures treated with different concentrations of LPS for 24 h and then exposed to 50 μM BSO for 48 h. Bars show Means ± SEM (N=4 per group; these experiments were repeated with similar results). ***$p<0.001$ compared to the corresponding control; ANOVA followed by Tukey test.

Figure 3. Treatment with LPS causes reduction in GSH levels in purified astrocytes and mesencephalic cultures. Astrocytes and mesencephalic cultures were exposed to 1 or 10 μg/ml LPS for 24 h and then treated with 10 μM BSO for an additional 48 h. LPS decreased GSH levels in astrocytes (A) and potentiated the depletion caused by BSO. In mesencephalic cultures (B) LPS lowered GSH content significantly, particularly at the higher concentration.

Mesencephalic cultures not treated with BSO had minimal cell survival and very low GSH levels. In the presence of 10 μg/ml LPS and BSO GSH levels were not detectable (ND), although there was no apparent damage to the cells. Bars show means ± SEM (N=4 per group) ***p<0.001 ANOVA followed by Tukey test.

Figure 4. The effect of growth factors on BSO toxicity. Mesencephalic cultures were treated with 50 ng/ml bFGF, TGF-β, or IGF-I for 24 h before exposure to BSO for an additional 48 h. Growth factors were present during BSO treatment. LDH released in the medium was expressed as percent of total LDH (medium + cells). Bars show means ± SEM (N=12, from 3 separate experiments). ***p<0.001; ANOVA followed by Tukey test.

Figure 5. The effect of IL-1β on BSO toxicity. Mesencephalic cultures were treated with 50 ng/ml IL-1β for 24 h before exposure to BSO for an additional 48 h. IL-1β was present during BSO treatment. LDH released in the medium was expressed as percent of total LDH (medium + cells). Bars show means ± SEM (N=10-12, from 3 separate experiments). ***p<0.001; **p<0.01; ANOVA followed by Tukey test.

Figure 6. LPS treatment up-regulates MnSOD in mesencephalic cultures. Cultures were treated with 10 μg/ml LPS for a total of 72h. (A) SOD activity, measured in triplicates from pooled cells, from 3 separate experiments. Bars show means ± SEM (N=3). *p<0.05 Student's t-test. (B) Western blots of lysates from control (lanes 1 and 3) and LPS (lanes 2 and 4) treated cultures from 2 independent experiments. Antibodies to tyrosine hydroxylase (TH) and MnSOD were applied to the same blot. Separate blots from the same lysates were used for Cu/ZnSOD.

Figure 7. Addition of SOD, but not catalase, protects mesencephalic cultures from BSO toxicity. Cultures were exposed to 100 units/ml catalase and/or 300 units/ml SOD for 24 h before treatment with 50 μM BSO. Fresh enzymes were added with BSO. MTT assay was performed 48 h after BSO treatment to determine cell viability. Bars show means ± SEM (N=6 from 2 independent experiments); ***$p<0.001$; ANOVA followed by Tukey test.

Figure 8. Immunocytochemical localization of Cu/ZnSOD in mesencephalic cultures. Control (upper panel) and LPS (10 μg/ml for 72h; lower panel) treated cultures were double labeled for Cu/ZnSOD (A, D) and MAP-2 (B, E). Overlay of the two images (C, F) shows predominantly neuronal localization of Cu/ZnSOD and no apparent changes after treatment with LPS. Bar=25 μm.

Figure 9. Immunocytochemical localization of MnSOD in mesencephalic cultures. Control and LPS (10 μg/ml for 72h; lower panel) treated cultures were double labeled for MnSOD and MAP-2 (A through F) and MnSOD and GFAP (G through L). In control cultures MnSOD (A) was expressed strongly in neurons (B), as shown in the overlay of the two images. LPS treatment caused a very strong increase in MnSOD immunoreactivity (D), which did not co-localize with MAP-2 positive neurons (E, F). Double label with GFAP showed MnSOD (G) present in some astrocytic processes (H, I). LPS treatment caused an increase in MnSOD (J), which was primarily within astrocytes (K, L). Bar = 25 μm.

Figure 1

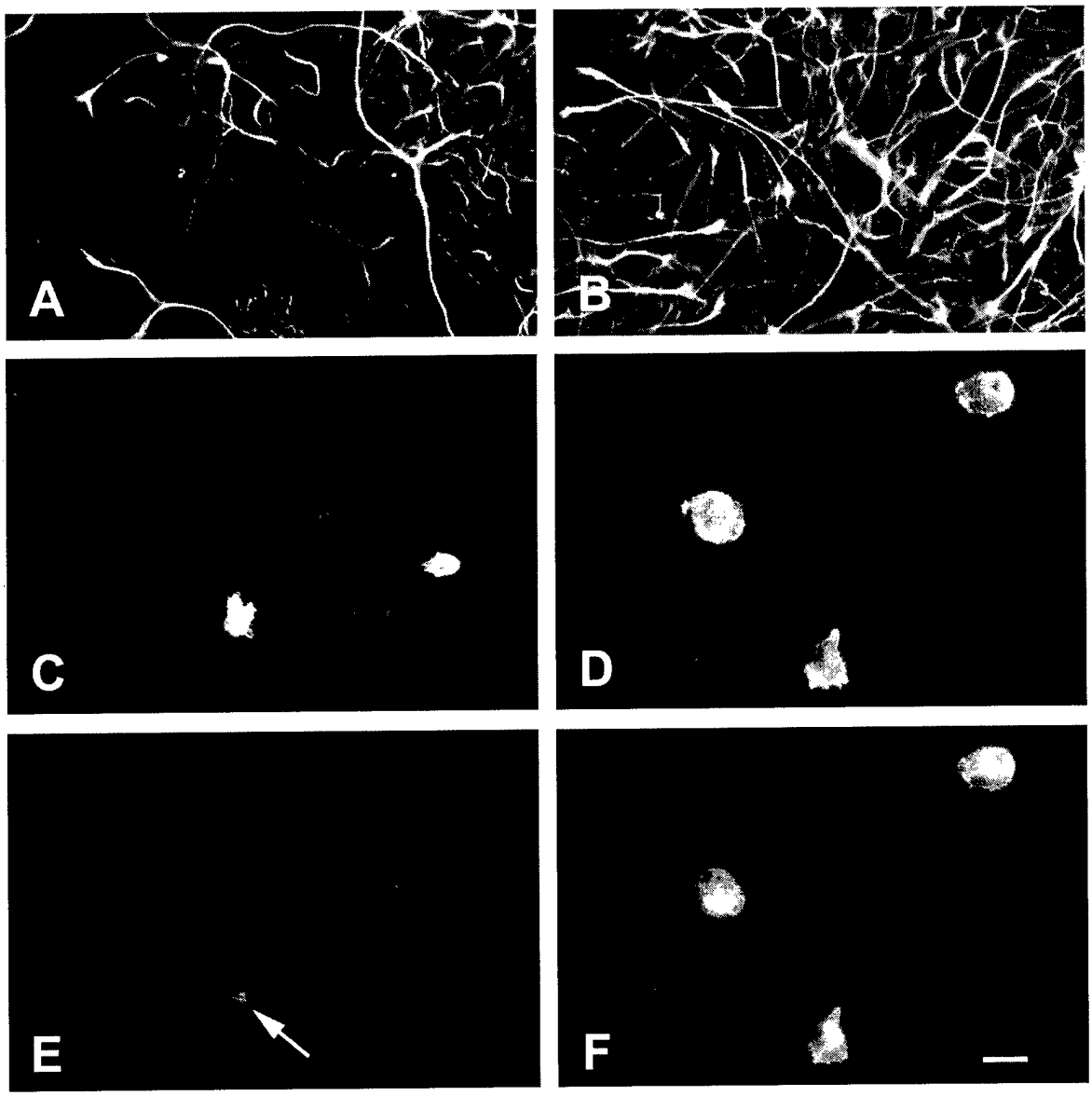


Figure 2

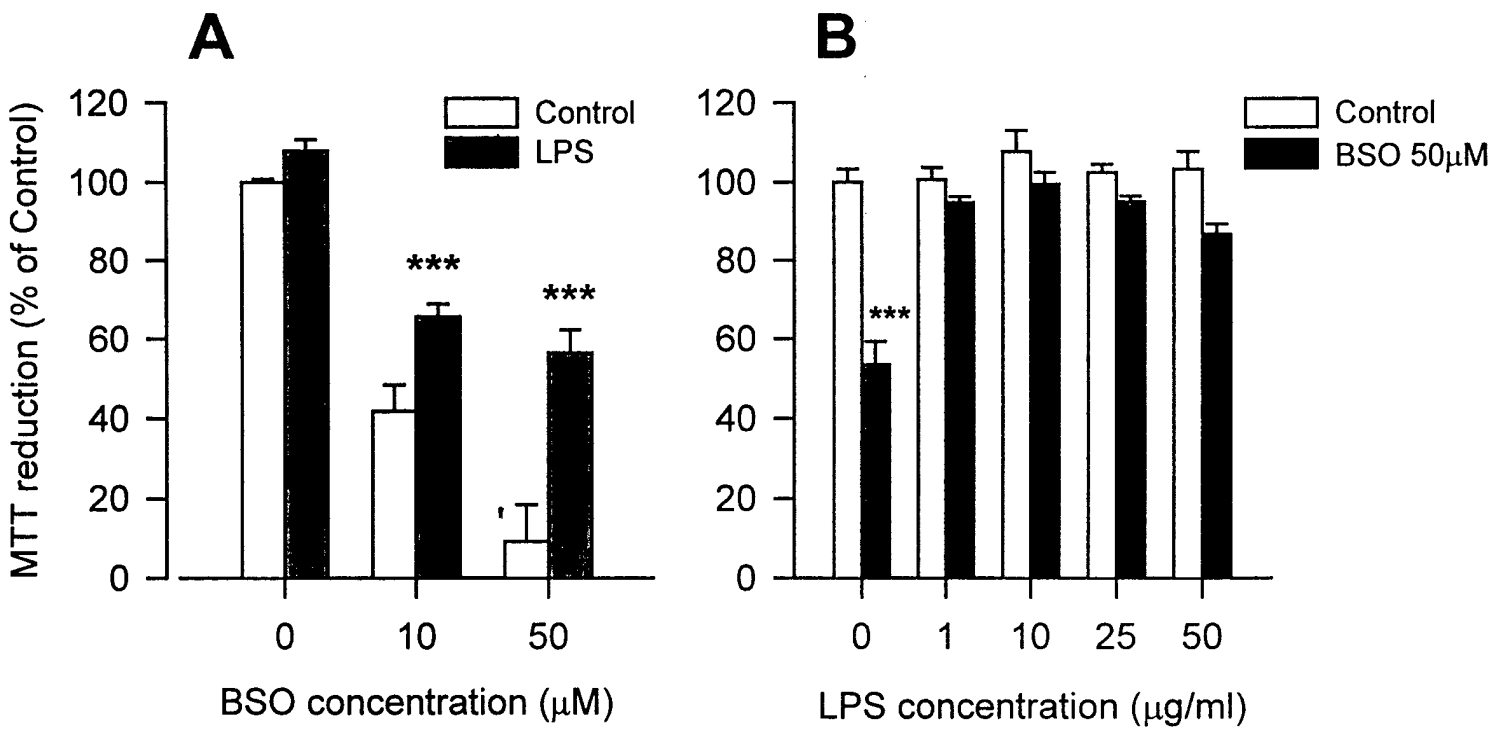
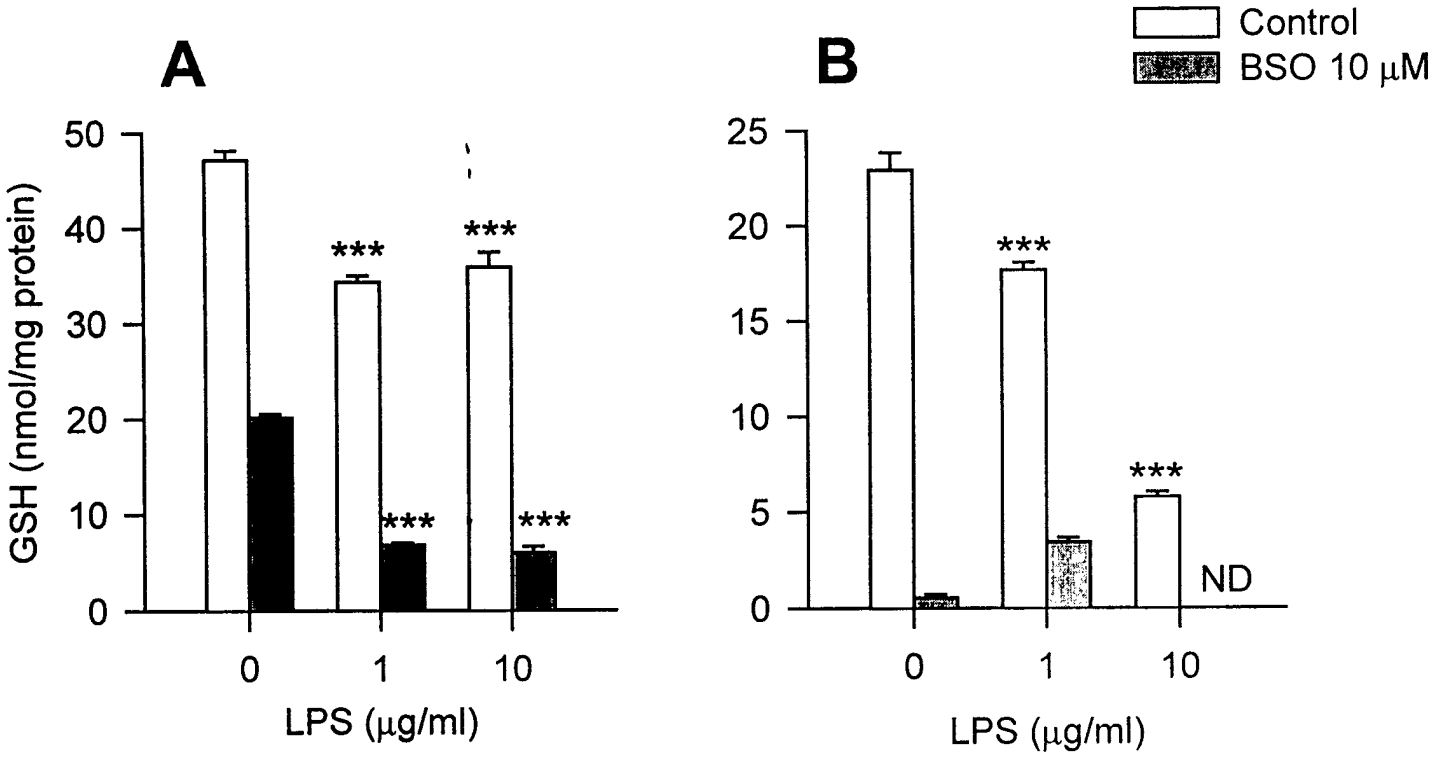

A
B
MTT reduction (% of Control)
120
100
80
60
40
20
0
Control
LPS
***
***
0
10
50
BSO concentration (μM)
Control
BSO 50μM
***
0
1
10
25
50
LPS concentration (μg/ml)

Figure 3


A
B
Control
BSO 10 μM
GSH (nmol/mg protein)
50
40
30
20
10
0
***
***
***
***
***
ND
0
1
10
LPS (μg/ml)
25
20
15
10
5
0
0
1
10
LPS (μg/ml)

Figuure 4

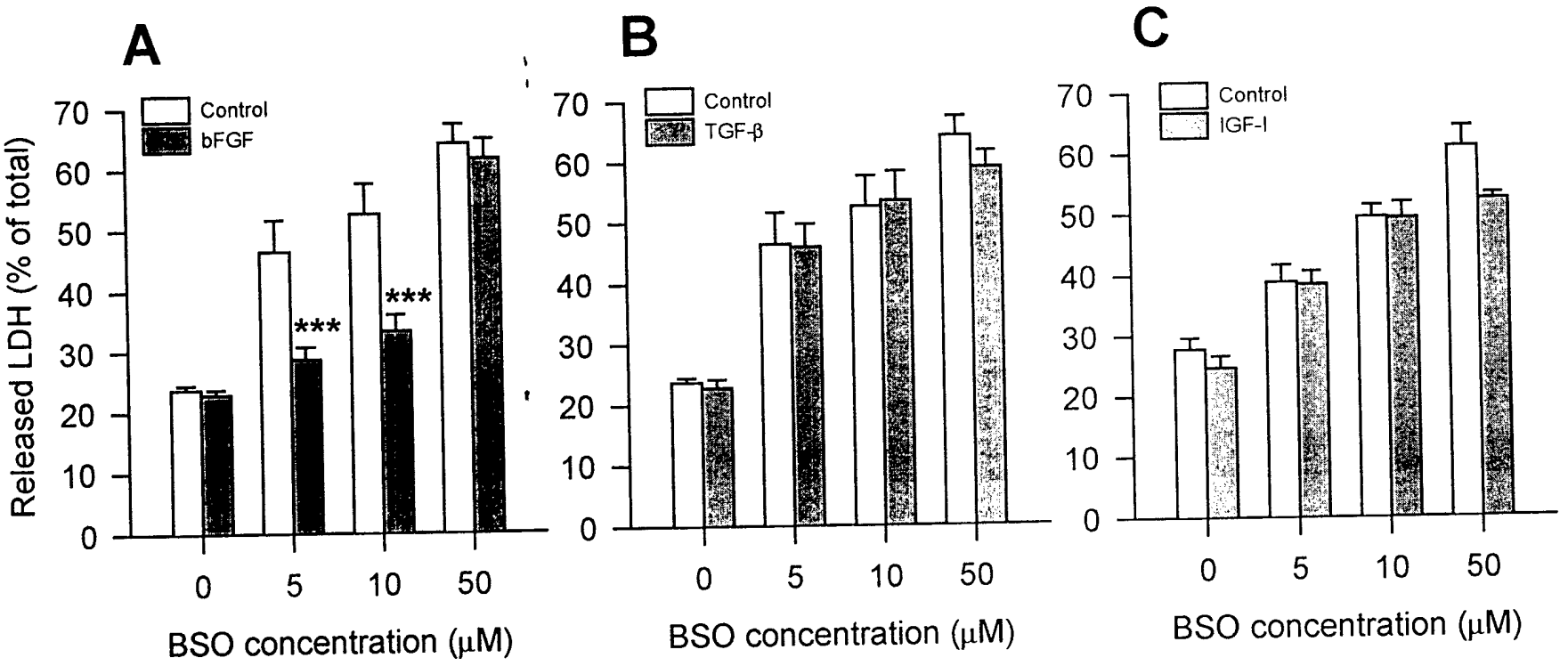

A
B
C
Control
bFGF
TGF-β
IGF-I
Released LDH (% of total)
70
60
50
40
30
20
10
0
***
***
0
5
10
50
BSO concentration (μM)

Figure 5

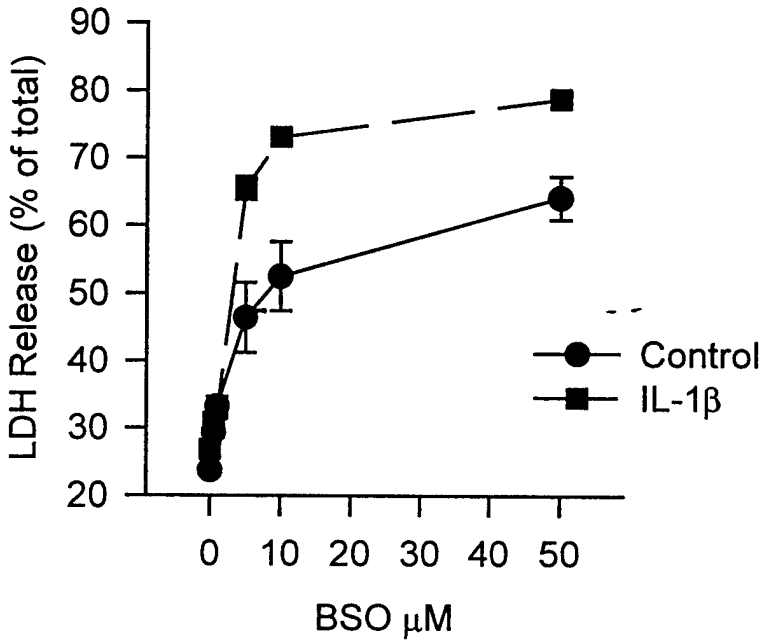

LDH Release (% of total)
90
80
70
60
50
40
30
20
0
10
20
30
40
50
BSO μM
Control
IL-1β

Figure 6

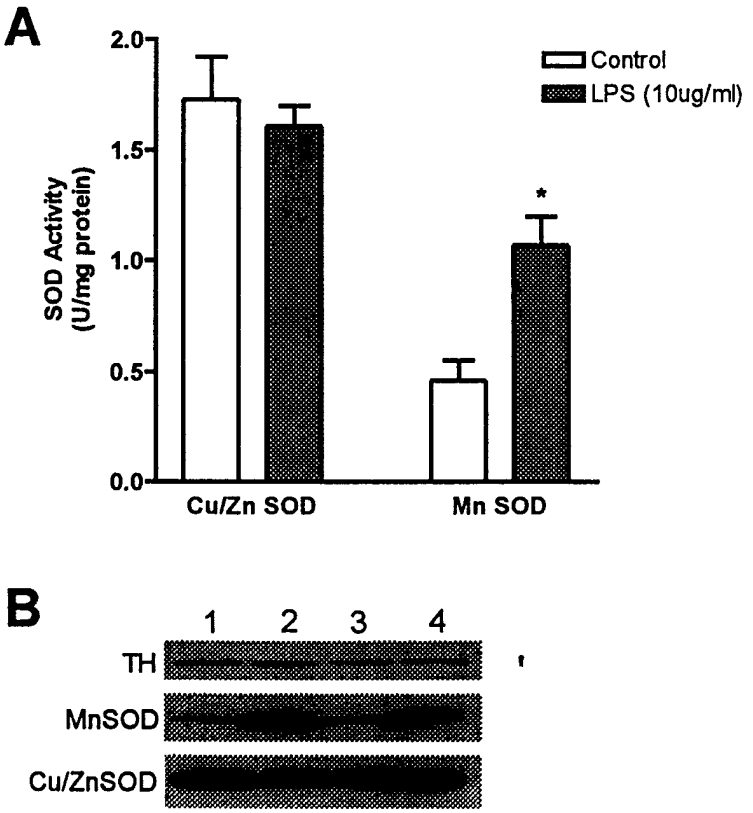

A
2.0
1.5
1.0
0.5
0.0
SOD Activity
(U/mg protein)
Control
LPS (10ug/ml)
*
Cu/Zn SOD
Mn SOD
B
1
2
3
4
TH
MnSOD
Cu/ZnSOD

Figure 7

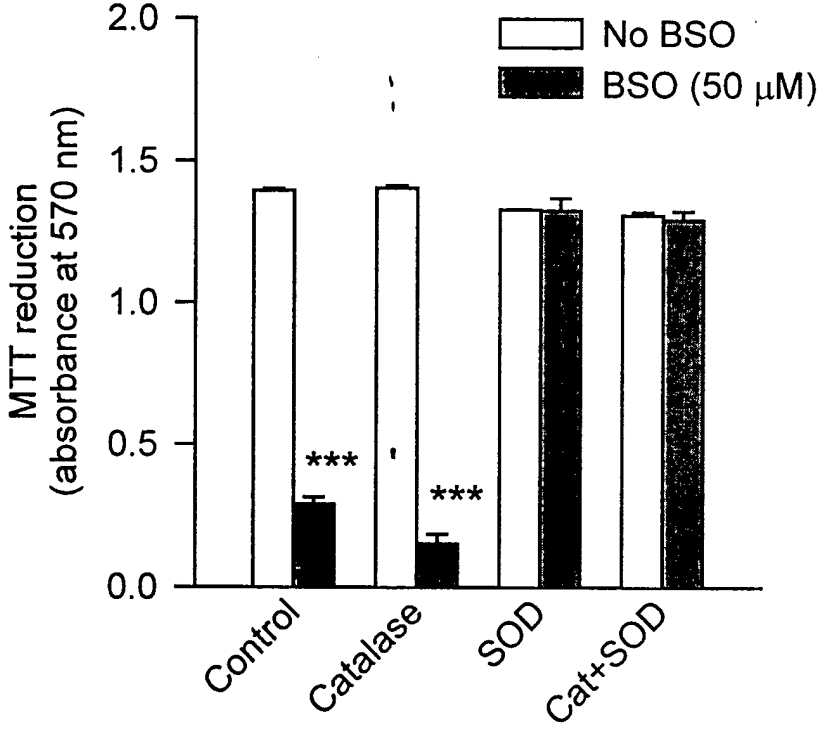

2.0
1.5
1.0
0.5
0.0
MTT reduction
(absorbance at 570 nm)
No BSO
BSO (50 μM)
***
***
Control
Catalase
SOD
Cat+SOD

Figure 8

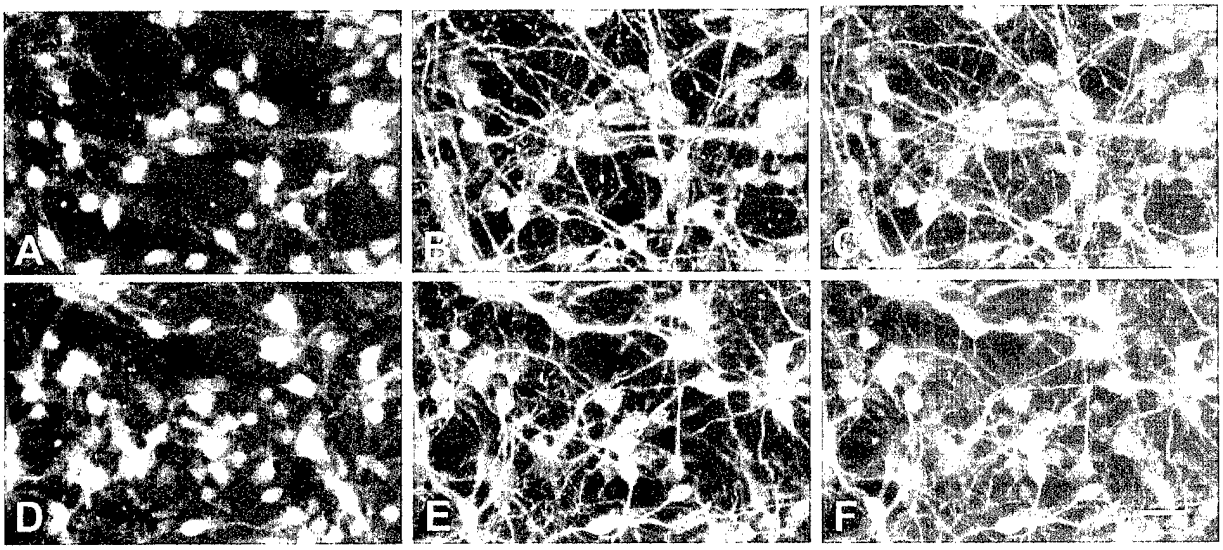


Figure 9